Bacteria of Dental Caries in Primary and Permanent Teeth in Children and Young Adults[▽]

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Although *Streptococcus mutans* has been implicated as a major etiological agent of dental caries, our cross-sectional preliminary study indicated that 10% of subjects with rampant caries in permanent teeth do not have detectable levels of *S. mutans*. Our aims were to use molecular methods to detect all bacterial species associated with caries in primary and permanent teeth and to determine the bacterial profiles associated with different disease states. Plaque was collected from 39 healthy controls and from intact enamel and white-spot lesions, dentin lesions, and deep-dentin lesions in each of 51 subjects with severe caries. 16S rRNA genes were PCR amplified, cloned, and sequenced to determine species identities. In a reverse-capture checkerboard assay, 243 samples were analyzed for 110 prevalent bacterial species. A sequencing analysis of 1,285 16S rRNA clones detected 197 bacterial species/phylotypes, of which 50% were not cultivable. Twenty-two new phylotypes were identified. PROC MIXED tests revealed health- and disease-associated species. In subjects with *S. mutans*, additional species, e.g., species of the genera *Atopobium*, *Propionibacterium*, and *Lactobacillus*, were present at significantly higher levels than those of *S. mutans*. *Lactobacillus* spp., *Bifidobacterium dentium*, and low-pH non-*S. mutans* streptococci were predominant in subjects with no detectable *S. mutans*. *Actinomyces* spp. and non-*S. mutans* streptococci were predominant in white-spot lesions, while known acid producers were found at their highest levels later in disease. Bacterial profiles change with disease states and differ between primary and secondary dentitions. Bacterial species other than *S. mutans*, e.g., species of the genera *Veillonella*, *Lactobacillus*, *Bifidobacterium*, and *Propionibacterium*, low-pH non-*S. mutans* streptococci, *Actinomyces* spp., and *Atopobium* spp., likely play important roles in caries progression.

Dental caries is one of the most common chronic infectious diseases in the world (2, 39). There are three major hypotheses for the etiology of dental caries: the specific plaque hypothesis, the nonspecific plaque hypothesis, and the ecological plaque hypothesis (24, 26, 37). The specific plaque hypothesis has proposed that only a few specific species, such as *Streptococcus mutans* and *Streptococcus sobrinus*, are actively involved in the disease. On the other hand, the nonspecific plaque hypothesis maintains that caries is the outcome of the overall activity of the total plaque microflora, which is comprised of many bacterial species (37). The ecological plaque hypothesis suggests that caries is a result of a shift in the balance of the resident microflora driven by changes in local environmental conditions (26).

Caries-associated bacteria traditionally have been identified by using culture-based methods, which exclude not-yet-cultivated species. Molecular methods for bacterial identification and enumeration now are performed routinely to more precisely study bacterial species that are associated with dental caries, including those that are not presently cultivable (32, 33).

In a previous study, Becker et al. (3) compared the bacterial species found in early childhood caries to those found in caries-free children. Some species, such as *Streptococcus sanguinis*, were associated with health, while others, such as *S. mutans*, other *Streptococcus* spp., *Veillonella* spp., *Actinomyces* spp., *Bifidobacterium* spp., and *Lactobacillus fermentum*, were associated with caries (3). These data also suggested that *Actinomyces gerencseriae* and other *Actinomyces* spp. play an important role in caries initiation. Munson et al. (29) used cultural and molecular techniques similar to ours to determine those species associated with the middle and advancing front of dental caries in adults. The authors demonstrated a diverse bacterial community, including *S. mutans*, *Lactobacillus* spp., *Rothia dentocariosa*, and *Propionibacterium* spp. (29). They also found that numerous novel taxa were present in carious lesions. Chhour et al. (8) used similar molecular techniques to determine the microbial diversity in advanced caries in adults. They demonstrated an abundance of species of the genera *Lactobacillus*, *Prevotella*, *Selenomonas*, *Dialister*, *Fusobacterium*, *Eubacterium*, *Olsenella*, *Bifidobacterium*, *Propionibacterium*, and *Pseudoramibacter*. *S. mutans* was not commonly detected. Corby et al. (10) examined the bacteria associated with dental caries and health in a subset of 204 twins aged 1.5 to 7 years old. A strain of an *Actinomyces* species, *S. mutans*, and *Lactobacillus* spp. were associated with disease. In contrast, bacterial species, including *Streptococcus parasanguinis*, *Abiotrophia de-*

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fectiva, *Streptococcus mitis*, *Streptococcus oralis*, and *S. sanguinis*, predominated in the indigenous bacterial flora of caries-free subjects (10). These findings concurred with those of our earlier study (1), demonstrating that there is a distinctive microbiota of the healthy oral cavity that is different from that associated with oral disease.

The purposes of this study were twofold: (i) to determine all bacterial species, cultivable as well as not yet cultivable, that are associated with health and dental caries of permanent teeth in children and young adults; and (ii) to describe the changes in bacterial profiles associated with the different states of this disease in primary and permanent teeth. Our major goal is to identify all of the species associated with health and disease, especially early on in the infection, that would provide alternative targets for biological intervention.

MATERIALS AND METHODS

Subject population. Subjects with severe dental caries and age-matched, caries-free controls were recruited from two different groups: (i) 15 subjects with caries in primary teeth and 14 controls and (ii) 36 subjects with caries in secondary teeth and 25 controls. Subjects were recruited from The Columbus Children's Hospital Dental Clinic, Columbus, OH, and The Ohio State University College of Dentistry Dental Clinic, Columbus. The subjects ranged in age from 2 to 21 years. Matching control subjects had no caries or existing restorations. Samples from primary teeth were previously analyzed (3) for 23 bacterial species or species groups. The actual samples were included in this study for comparisons and for analysis in an expanded panel of 110 bacterial species.

Sampling. Plaque samples from the 39 healthy subjects were pooled from a minimum of four sites, including anterior and posterior teeth. From the 51 caries subjects, plaque was collected separately from each of the following four different types of sites: (i) surfaces of intact enamel, (ii) surfaces of white-spot lesions, (iii) cavitated dentin lesions, and (iv) deep-dentin lesions. From the enamel, white-spot lesions, and cavitated lesions, the plaque was obtained by swiping the tooth surface with a dental explorer and transferring it onto a coarse sterile endodontic paper point. The carious dentin was excavated with either a spoon excavator or a round burr at slow speed using a dental drill. Each sample was obtained by pooling material collected from at least three different teeth. Samples were placed in a sterile 1.5-ml microcentrifuge tube and transported to the laboratory, where they were frozen until further analysis. Samples were collected in a cross-sectional fashion, representing all of the defined disease states, to describe the changes in the bacterial profiles of predominant bacteria.

Isolation of bacterial DNA. The DNA was isolated (3) and purified (22) as previously described and frozen at −20°C for later analysis.

Clonal analysis. The samples from permanent teeth were selected for bacterial clonal analysis: three caries subjects with detectable levels of *S. mutans*, two caries subjects without detectable levels of *S. mutans*, and two subjects from the healthy control group. Each of the disease states (as described above) was represented among the five selected caries subjects and one pooled sample from the healthy subjects, for a total of 22 samples. The selection of subjects for clonal analysis was based on results from preliminary checkerboard analyses (see below).

Amplification of 16S rRNA genes by PCR and purification of PCR products. The 16S rRNA gene was amplified using a universal primer set (1) under standardized conditions, and the purification of PCR products was performed as previously described (1).

Cloning procedures. The cloning of PCR-amplified DNA was performed with the TOPO TA cloning kit (Invitrogen, San Diego, CA) according to the instructions of the manufacturer. Correct sizes of the inserts were determined in a PCR with an M13 (−20) forward primer and an M13 reverse primer (Invitrogen). Prior to the sequencing of the fragments, the PCR-amplified 16S rRNA gene fragments were purified and concentrated according to the methods of Paster et al. (33).

16S rRNA gene sequencing. Purified PCR-amplified 16S rRNA gene inserts were sequenced using an ABI Prism cycle sequencing kit (BigDye Terminator cycle sequencing kit with AmpliTaq DNA polymerase FS; ABI, Foster City, CA) and a GeneAmp PCR system 9700 (ABI). The primers and sequencing reactions used for sequencing have been described previously (33).

16S rRNA gene sequencing and data analysis of unrecognized inserts. Inserts of the correct size of approximately 1,500 bases were analyzed from 46 to 70

clones per sample (an average of 58.4 clones with a standard deviation of 6.3) for a total of 1,285 clones. Healthy controls and each of the four disease states (described above) were represented in the 22 samples analyzed. A sequence of approximately 500 bases was obtained first to determine the identity or approximate phylogenetic position. Full sequences of about 1,500 bases were achieved by using five to six additional sequencing primers (19) for those species deemed novel. Sequences of inserts were analyzed according to Aas et al. (1). The similarity matrices were corrected for multiple-base changes at single positions (18) and were constructed from the aligned sequences by using only those sequence positions for which data were available for 90% of the strains tested. The construction (34) and drawing (38) of phylogenetic trees were done according to Paster et al. (33). We are aware of the potential creation of 16S rRNA gene chimera molecules assembled during the PCR (23). The percentage of chimeras in 16S rRNA gene libraries ranged from 1 to 15%. Chimeric sequences were identified by using the Chimera Check program in The Ribosomal Database Project (RDP-II; release 9.55; Center for Microbial Ecology, Michigan State University; http://rdp.cme.msu.edu/), by treeing analysis, or by base signature analysis. The species identification of the chimeras was obtained, but the sequences were not examined for the phylogenetic analysis.

Reverse-capture checkerboard assay. Based on the sequencing data from clonal analyses and previous results (3), 16S rRNA gene-based probes were designed for the most prevalent bacterial species associated with caries. Sequences selected for use as probes were identified by the alignment of 16S rRNA gene sequences to detect regions where mismatches occurred (32). 16S rRNA gene probes were designed for a hybridization temperature of 55°C, and the standard probe length was 18 to 20 nucleotides. New probes were quality tested against an extended clone library for cross-reactivity. From the 90 subjects, 1,000 clinical samples were analyzed for 110 bacterial supragingival species in a DNA-DNA hybridization assay (Table 1). The reverse-capture checkerboard assay was carried out as previously described (3, 32). DNA probes were synthesized with multiple thymidines (T) at the 5′ end of the oligonucleotide. The poly(T) tails were cross-linked to a nylon membrane support via UV irradiation, leaving the specific probe available for hybridization. The 16S rRNA genes from clinical samples were amplified by PCR with universal primers; the forward primer was labeled at the 5′ end with digoxigenin. The digoxigenin-labeled amplicon was hybridized to the capture probes bound to the membrane, and the digoxigenin residues were complexed to anti-digoxigenin antibody covalently bound to alkaline phosphatase. The levels of chemifluorescence were measured using a Storm system (Storm Technology Inc., Mountain View, CA).

Statistical analysis. Bacterial signals were measured by the intensity of spots on checkerboard membranes with ImageQuant TL (Amersham Biosciences, Piscataway, NJ) for array analysis as previously described (10). Statistical comparisons were made by pairwise comparisons of the healthy controls to each of the sample groups (intact enamel, white-spot lesions, cavitated dentin lesions, and deep-dentin lesions) in subjects with caries by the PROC MIXED procedure (SAS, version 8; SAS Institute Inc., Cary, NC). The variance between the disease states is defined with the random statement of disease and dentition association for each of the bacteria tested (data not shown).

Nucleotide sequence accession numbers. The complete 16S rRNA gene sequences of clones representing novel phylotypes defined in this study, sequences of known species not previously reported, and published sequences are available for electronic retrieval from the EMBL (http://www.ebi.ac.uk/embl/), GenBank (http://www.ncbi.nlm.nih.gov/GenBank/), and DDBJ (http://www.ddbj.nig.ac.jp/) nucleotide sequence databases under the accession numbers shown in Fig. 1, 2, and 3.

RESULTS AND DISCUSSION

In our preliminary study, supragingival plaque from each of the four defined disease states in 42 subjects with severe dental caries in the secondary dentition was analyzed, screening for a selection of 28 caries-associated bacterial species using a reverse-capture DNA-DNA hybridization assay. The results of the study demonstrated that 10% of subjects with rampant caries in the secondary dentition do not have detectable levels of *S. mutans*. In that study, *S. mutans* was not detected in intact enamel and white-spot lesions of diseased subjects, while most dentin lesions or deep-dentin lesions harbored *S. mutans*.

The second step of the study was to assess those bacterial

TABLE 1. 16S rRNA gene probes used in a reverse-capture checkerboard assay to detect predominant species and groups of species of bacteria[a]

Probe designation	Probe designation
Abiotrophia defectiva	*Lactobacillus* sp. cl. CX036
Actinomyces georgiae	*Lautropia mirabilis*
Actinomyces gerencseriae	*Leptotrichia* all
Actinomyces israelii	*Leptotrichia* sp. cl. BU064
Actinomyces naeslundii serotype II	*Leptotrichia* sp. cl. DE081
Actinomyces odontolyticus sp. cl. DR002	*Leptotrichia* sp. cl. DR011
Actinomyces sp. GU067	*Leptotrichia* sp. cl. DS049
Actinomyces sp. oral cl. AG004	*Leptotrichia* sp. cl. DT031
Actinomyces sp. oral cl. AP064	*Leptotrichia* sp. cl. GT020
Actinomyces sp. oral cl. EP005	*Leptotrichia* sp. cl. GT018
Actinomyces sp. strain ATCC 49338	*Leptotrichia* sp. cl. A39FD
Actinomyces sp. strain B19SC	*Neisseria mucosa*/*Neisseria flavescens*
Actinomyces sp. strain B27SC	*Peptostreptococcus* sp. cl. CK035
Atopobium genomospecies C1	*Porphyromonas* sp. cl. DS033
Bacteroides sp. cl. AU126	*Prevotella* sp. cl. GU069/*Prevotella* sp. cl. GU027
Bifidobacterium all[b]	*Prevotella loescheii*
Bifidobacterium sp. oral cl. CX010	*Prevotella melaninogenica*
Campylobacter concisus	*Prevotella oris*
Campylobacter gracilis	*Prevotella* sp. cl. AO036
Campylobacter showae	*Propionibacterium* sp. strain FMA5
Capnocytophaga gingivalis	*Rothia dentocariosa*
Capnocytophaga granulosum	*Selenomonas infelix*
Capnocytophaga ochracea ρ 089	*Selenomonas noxia*
Capnocytophaga sp. cl. DS022	*Selenomonas* sp. cl. AA024
Capnocytophaga sputigena	*Selenomonas* sp. cl. GT010
Cardiobacterium sp. strain A/*Cardiobacterium hominis*	*Selenomonas* sp. cl. GT052
Cardiobacterium sp. cl. BU084/BM058	*Selenomonas* sp. cl. EY047
Catonella morbi	*Selenomonas* sp. oral cl. Cs024
Corynebacterium matruchotii	*Selenomonas* sp. caries cl. DS051
Corynebacterium sp. cl. AK153	*Selenomonas* sp. caries cl. DS071
Eubacterium saburreum sp. cl. GT038	*Selenomonas sputigena*
Eubacterium sp. cl. BB124	*Streptococcus anginosus*
Eubacterium sp. cl. BE088	*Streptococcus australis* T1-E5
Eubacterium sp. cl. C27KA	*Streptococcus cristatus*
Eubacterium sp. cl. DO008	*Streptococcus gordonii*
Eubacterium sp. cl. DO016	*Streptococcus intermedius*
Eubacterium sp. cl. EI074	*Streptococcus mitis*
Eubacterium yurii	*Streptococcus mitis* biovar 2
Fusobacterium all	*Streptococcus mitis*/*Streptococcus oralis*
Fusobacterium animalis	*Streptococcus mutans*
Fusobacterium nucleatum subsp. *vincentii*	*Streptococcus parasanguinis*
Fusobacterium periodonticum	*Streptococcus salivarius*
Fusobacterium nucleatum subsp. *polymorphum*	*Streptococcus sanguinis*
Gemella haemolysans	*Streptococcus* sp. cl. CH016
Gemella morbillorum	*Streptococcus* sp. group H6
	Streptococcus sp. oral cl. AA007
Granulicatella adiacens	*Streptococcus* sp. strain 7A/*Streptococcus* sp. strain H6
Haemophilus parainfluenzae	*Streptococcus* sp. strain T4-E3
Kingella sp. cl. DE012/*Neisseria polysaccharea*	*Tannerella forsythia*-like cl. BU063
Kingella denitrificans	*Treponema socranskii* subsp. *paredis*
Kingella oralis	*Veillonella atypica*
Lactobacillus all	*Veillonella dispar*/*parvula*
Lactobacillus fermentum	*Veillonella* sp. oral cl. BU083
Lactobacillus gasseri	

[a] Combination probes are named after the pairs of species (e.g., "*Cardiobacterium* sp. strain A/*Cardiobacterium hominis*") or groups of species (e.g., "*Bifidobacterium* all") of bacteria detected.
[b] all, all species of the genus.

species associated with the progression of dental caries in permanent teeth using pooled samples representing the disease states; intact enamel, white-spot lesions, dentin lesions, and deep-dentin lesions in each of the five subjects with severe caries and pooled plaque from two healthy controls were selected for cloning and sequencing. Samples from subjects with and without detectable levels of *S. mutans* were included for

comparison, and the selection of subjects for the clonal analysis was based on results from preliminary checkerboard analyses. Sequences from a total of 1,285 16S rRNA gene clones were used to identify species and the closest relatives in 22 samples. Sequence analysis demonstrated a striking bacterial diversity of 197 species or phylotypes, representing eight bacterial phyla. Of this total, 99 (50%) strains have not yet been cultivated, 12

w Sm w Sm w Sm w/o Sm w/o Sm

1 2 a b c d a b c d a b c d a b c d a b c d

5%

Streptococcus mutans; AJ243965
Streptococcus anginosus; AF104678
Streptococcus intermedius; AF104671
Streptococcus constellatus; AF104676
Streptococcus salivarius; M58839
Streptococcus cristatus; AB008313
Streptococcus gordonii; AF003931
Streptococcus sp. clone AG008; AY134909
Streptococcus parasanguinis; X53652
Streptococcus australis; AF385525
Streptococcus sanguinis; AF003928
Streptococcus sp. strain 7A; AY005040
Streptococcus sp. strain 12F; AY134908
Streptococcus sp. clone CH016; AY005044
Streptococcus sp. clone BW009; AY005042
Streptococcus sp. strain H6; AY005041
Streptococcus mitis biovar2; AY005045
Streptococcus mitis; AF003929
Streptococcus sp. clone EK048; AF385574
Abiotrophia defectiva; D50541
Granulicatella adiacens; D50540
Lactobacillus casei; X61135
Lactobacillus sp. TMV1.666; AJ250074
Lactobacillus sp. clone CX036; AY005048
Lactobacillus fermentum; AB017345
Lactobacillus ultunense; AY253660
Lactobacillus sp. clone HT002; AY349382
Lactobacillus gasseri; M58820
Gemella haemolysans; M58799
Gemella morbillorum; L14327
Eubacterium saburreum; AF287777
Eubacterium sp. clone IR009; AY349376
Eubacterium sp. clone EI074
Lachnospiraceae sp. clone DO016; AF385510
Lachnospiraceae sp. clone AO068; AF287771
Lachnospiraceae sp. MCE9_31; AF481220
Lachnospiraceae sp. clone CK035; AF287763
Eubacterium brachy; U13038
Veillonella sp.clone AA050; AF287782
Veillonella parvula dispar; X84005/X84006
Veillonella atypica; X84007
Megasphaera sp. clone BS073; AF287785
Dialister invisus; AF287788
Selenomonas sputigena; AF287793
Selenomonas sp. clone CS002; AF287795
Selenomonas sp. clone AA024; AF287797
Selenomonas sp. clone EY047;
Selenomonas noxia; AF287799
Olsenella genomosp. C1; AY278623
Atopobium rimae; X67149
Bifidobacterium dentium; M58735
Corynebacterium sp. clone AK153; AF287755
Corynebacterium matruchotii; X82065
Actinobaculum sp. clone EL030; AY008311
Actinomyces sp. strain Hal-1083; AF385522
Actinomyces odontolyticus; AJ234040
Actinomyces meyeri; X82451
Actinomyces sp. strain B27SC; AF287750
Actinomyces graevenitzii; AJ540309
Actinomyces sp. clone EP053; AY008316
Actinomyces israelii; X82450
Actinomyces gerencseriae; X80414
Actinomyces sp. clone IP073; AY349365
Actinomyces sp. clone IP081; AY349366
Actinomyces sp. clone AP064; AF2877748
Actinomyces sp. clone IO077; AY349364
Actinomyces sp.clone GU067; AY349362
Actinomyces sp. clone EP005; AY008314
Actinomyces sp. clone AG004; AF287747
Actinomyces naeslundii II; X53225
Actinomyces naeslundii CCUG 34286; AJ234051
Fusobacterium sp. clone BS019; AF366265
Fusobacterium nucleatum ss *polymorphum*; AF287812
Fusobacterium nucleatum ss *animalis*; X55404
Fusobacterium nucleatum ss *vincentii*; AJ006965
Leptotrichia sp. clone DR011; AF385518
Leptotrichia sp. clone BU064; AY008309
Leptotrichia sp. clone IK040; AY349387
Leptotrichia wadei; AY029807
Leptotrichia buccalis; X90831
Campylobacter showae; L06974
Campylobacter gracilis; L04320
Campylobacter concisus; L06977
Neisseria mucosa; AJ247257
Burkholderia cepacia; U96927
Lautropia sp. clone FX006; AY134902
Lautropia mirabilis; X97652
Lautropia sp. clone AP009; AY005030
Capnocytophaga sp. clone BM058; AY005075
Capnocytophaga sp. clone BU084; AF385569
Capnocytophaga sputigena; L14636
Tannerella sp. clone BU063; AY008308
Bacteroidetes sp. clone AU126; AY005072
Porphyromonas sp. clone CW034; AY008310
Prevotella sp. clone CY006; AY005063
Prevotella oris; L16474
Prevotella sp. clone BE073; AF385551
Prevotella dentalis; X81876
Prevotella sp. strain B31FD; AY005063
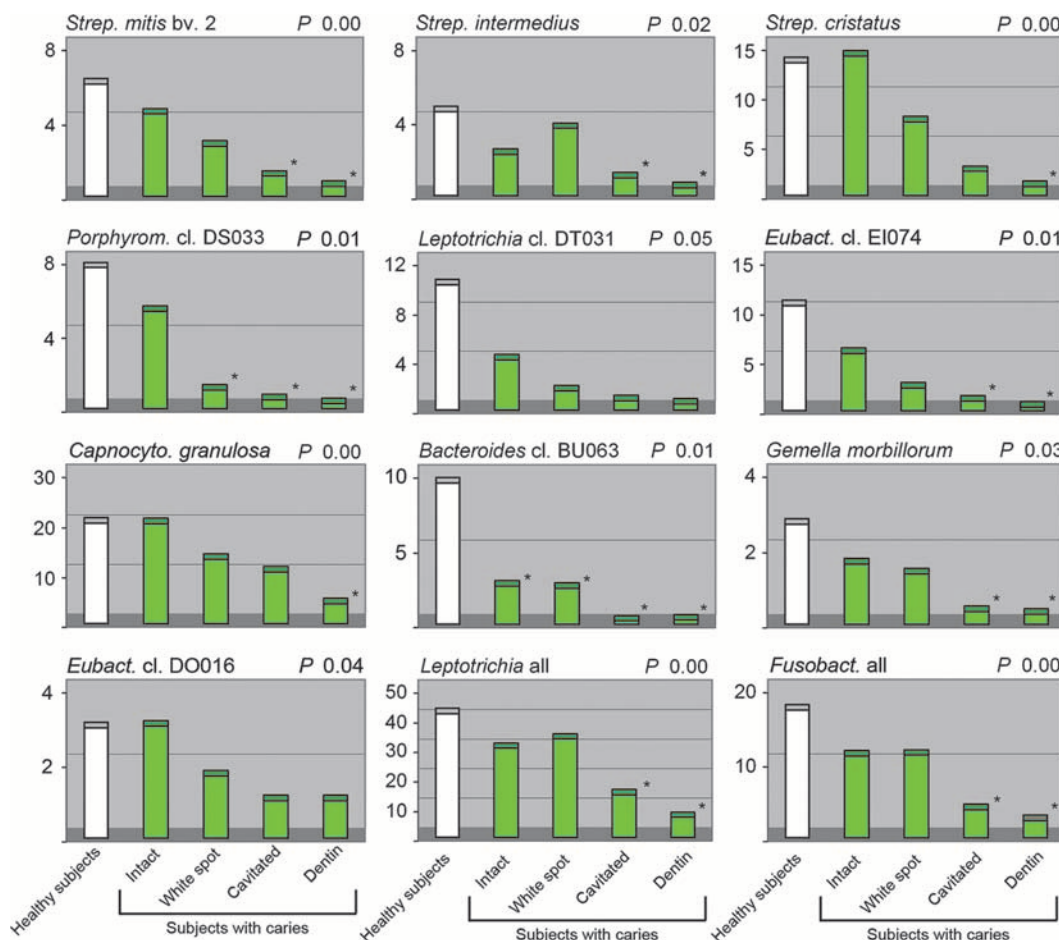*Prevotella loescheii*; L16481

FIG. 2. Mean levels of health-associated species or species groups in primary teeth determined by a reverse-capture checkerboard assay for 15 subjects with caries (green bars) and 14 healthy controls (white bars). *, $P \le 0.05$ for pairwise comparisons of the healthy group to other groups by PROC MIXED. *P* indicates the overall *P* value comparing all groups and sites by PROC MIXED. *Strep.*, *Streptococcus*; *Porphyrom.*, *Porphyromonas*; *Eubact.*, *Eubacterium*; *Capnocyto.*, *Capnocytophaga*; *Fusobact.*, *Fusobacterium*; cl., clone.

were previously uncharacterized strains, and 86 were known species or phylotypes. Twenty-two novel phylotypes were identified in this study. From each lesion, the number of taxa ranged from 8 to 37 (Fig. 1). The phylogenetic relationships of species detected in health and disease are shown in Fig. 1, and the differences in bacterial profiles between healthy samples and those from each of the four stages of the disease are summarized in Table 2.

In subjects with *S. mutans* (subjects 1, 2, and 3) (Table 2), additional species, such as *Atopobium* genomospecies C1 or *Lactobacillus* spp., were present at significantly higher levels. In subject 1, *Atopobium* genomospecies C1 predominated among all species, constituting 40% of the clones, while *S. mutans* was detected only at a low level (Fig. 1). In subjects without *S. mutans*, subject 4 had a predominance of *Lactobacillus* spp.,

whereas subject 5 had a predominance of *Bifidobacterium dentium* and low-pH non-*S. mutans* streptococci, including *S. mitis*, *S. sanguinis*, *Streptococcus gordonii*, *Streptococcus* sp. clone BW009, *Streptococcus* sp. clone EK048, and *Streptococcus salivarius* (Fig. 1). The bacterial profiles during the initiation of caries (i.e., on intact enamel and white-spot lesions) (Fig. 1, sites a and b) were more distinct and complex than those in established caries (i.e., dentin and deep-dentin lesions) (Fig. 1, sites c and d, and Table 2). *Actinomyces* spp. and non-*S. mutans* streptococci were detected at high levels in caries initiation (Fig. 1, sites a and b, and Table 2) compared to those of other disease states. Bacterial profiles of caries in the secondary dentition varied from subject to subject (Fig. 1, Table 2).

The goals of the clonal analyses were to first determine the breadth of bacterial diversity in dental plaque samples and

FIG. 1. Phylogenetic relationships of species detected from dental caries in permanent teeth and from healthy controls. Subjects with (w *Sm*) and without (w/o *Sm*) detectable levels of *S. mutans* (*Sm*) were selected for clonal analysis. The distribution and levels of bacterial species/ phylotypes among the five subjects and two healthy controls are shown by the columns of boxes to the right of the tree as either not detected (clear box), <15% of the total number of clones assayed (shaded box), or ≥15% of the total number of clones assayed (darkened box). Fifteen percent was chosen as the arbitrary level. GenBank accession numbers are provided. The marker bar represents a 5% difference in nucleotide sequences.
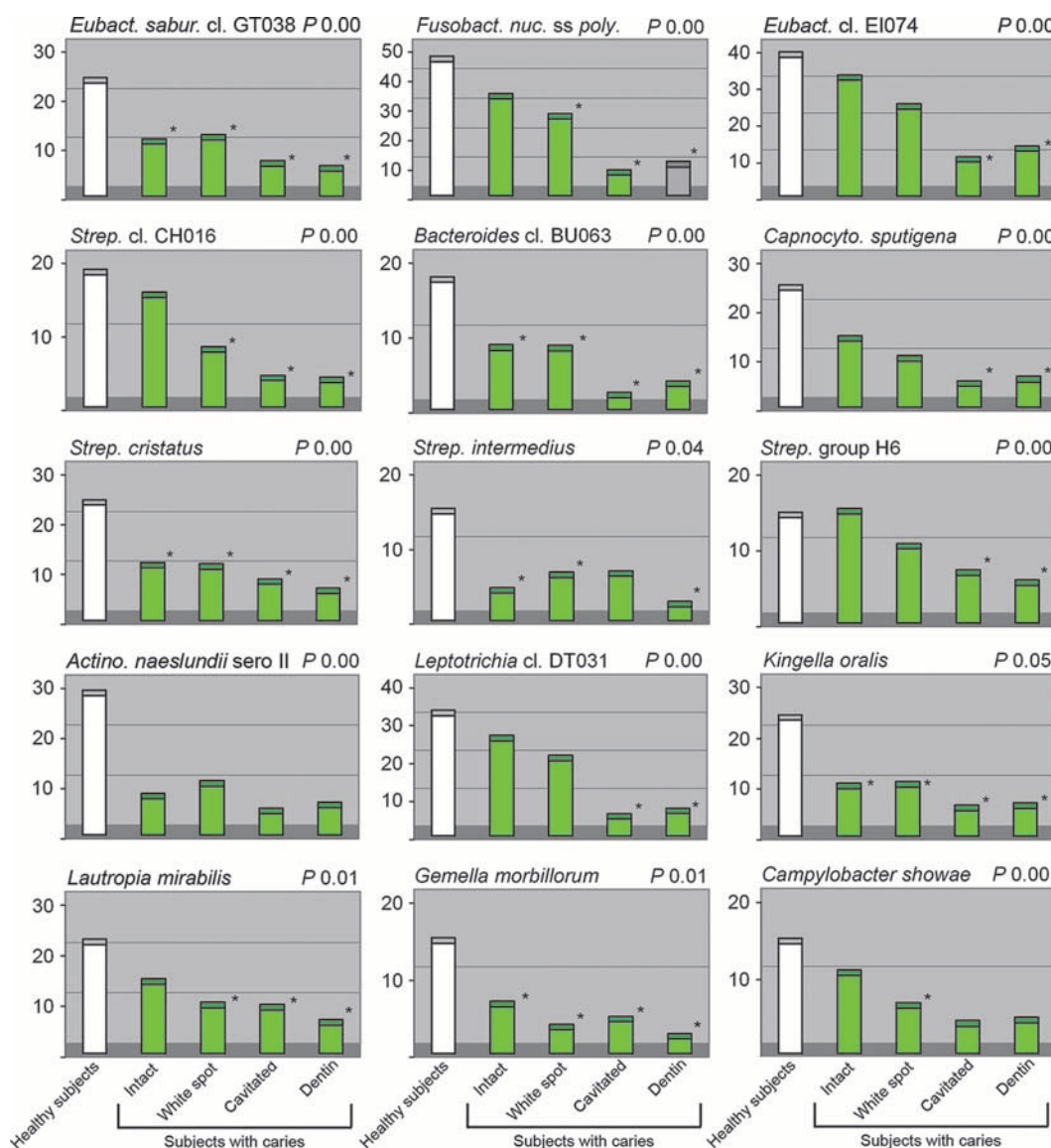
FIG. 3. Mean levels of health-associated species or species groups in permanent teeth determined by a reverse-capture checkerboard assay for 36 subjects with caries (green bars) and 25 healthy controls (white bars). ∗, $P \leq 0.05$ for pairwise comparisons of the healthy group to other groups by PROC MIXED. P indicates the overall P value comparing all groups and sites by PROC MIXED. *Strep.*, *Streptococcus*; *Eubact.*, *Eubacterium*; *Eubact. sabur.*, *Eubacterium saburreum*; *Capnocyto.*, *Capnocytophaga*; *Fusobact. nuc.* ss *poly.*, *Fusobacterium nucleatum* subsp. *polymorphum*; *Actino*, *Actinomyces*; cl., clone.

then assess specific bacterial associations with health and disease. However, to better assess bacterial associations with health status, the final step of our study was to use a reverse-capture DNA-DNA hybridization assay using oligonucleotide probes targeting 110 prevalent oral bacterial taxa. A total of 243 clinical samples from primary and secondary teeth in a total of 90 selected subjects were analyzed. Bacteria in pooled plaque samples from the enamel of healthy subjects ($n = 39$) were compared to those in samples from intact enamel, white-spot lesions, and dentin and deep-dentin lesions from each of the subjects with severe decay ($n = 51$). Pooled plaque samples from a minimum of four sites in healthy individuals and pooled material collected from at least three different teeth representing four states of caries may dilute the number of bacteria that

dominate and alter the proportion of species present at a single site. Nevertheless, the predominant species present still will be detected.

Based on these checkerboard analyses, differences in bacterial profiles between primary and secondary teeth, as well as those of a number of health-associated and disease-associated species, were revealed (Fig. 2 to 5, Table 3). Statistical analyses also showed that bacterial profiles of the intact enamel of healthy subjects differed significantly from the bacterial profiles of intact enamel from diseased subjects in both age groups (data not shown). In primary teeth, health-associated species such as *Capnocytophaga granulosa*, *Eubacterium* sp. clone DO016, and *Streptococcus cristatus* were detected at the same levels in plaque from intact enamel of diseased subjects and

TABLE 2. Progression of dental caries

Species	Healthy subject no.		Diseased subject no.																			
			1 (with *S. mutans*)				2 (with *S. mutans*)				3 (with *S. mutans*)				4 (without *S. mutans*)				5 (without *S. mutans*)			
	1	2	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
Streptococcus mutans	−	−	+	+	−	+	−	−	**+**	**+**	−	−	+	+	−	−	−	−	−	−	−	−
Abiotrophia and *Granulicatella* spp.	−	+	−	−	−	−	**+**	**+**	**+**	−	−	+	−	−	−	−	−	−	**+**	+	+	+
Actinomyces spp.	+	+	**+**	**+**	−	+	−	−	−	−	+	**+**	−	−	+	+	−	−	**+**	**+**	+	−
Actinobaculum spp.	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Atopobium spp.	−	−	−	−	−	−	−	−	+	−	−	−	−	**+**	−	−	−	−	−	−	−	−
Bifidobacterium dentium	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
Capnocytophaga spp.	−	−	−	−	−	−	−	−	−	−	**+**	−	−	−	−	−	−	−	−	−	−	−
Corynebacterium spp.	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Eubacterium spp.	−	+	−	−	**+**	+	−	−	−	+	**+**	−	**+**	+	−	−	−	−	−	−	−	−
Fusobacterium spp.	+	−	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	+	−	−
Lactobacillus spp.	−	−	−	−	−	−	−	−	**+**	**+**	−	−	−	**+**	−	−	+	+	−	+	−	−
Leptotrichia spp.	+	+	+	−	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Prevotella spp.	−	+	−	−	−	**+**	**+**	+	−	−	+	**+**	−	−	−	−	−	−	−	−	−	−
Selenomonas spp.	+	+	−	−	−	+	−	−	−	−	+	−	+	−	−	−	−	−	+	−	+	−
Streptococcus anginosus	−	−	**+**	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Streptococcus gordonii	−	−	**+**	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
Streptococcus intermedius	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	**+**	−	−	−	−	−	−
Streptococcus parasanguinis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	**+**	+	+	+	−	−	−	−
Streptococcus salivarius	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+
Streptococcus sanguinis	−	+	−	−	−	−	−	−	−	−	**+**	+	+	+	−	−	−	−	+	+	+	+
Veillonella spp.	+	+	+	+	+	+	−	−	+	−	+	+	+	+	**+**	**+**	**+**	+	+	+	+	+

[a] The presence (+) or absence (−) of predominant species at specific sites is indicated. Boldface indicates species detected in >15% of the total number of clones. a, intact enamel; b, white spots; c, dentin cavities; d, deep-dentin cavities.

plaque from healthy subjects (Fig. 2). Even though *S. mutans* was barely present in the permanent teeth of healthy subjects, it was well represented in the bacterial flora of the healthy enamel of diseased subjects (Fig. 5). The bacterial flora of healthy sites in primary teeth was characterized by 15 significant health-associated species, which was in contrast to the 30 health-associated species that we found at healthy sites in permanent teeth. As many as 13 of the 15 significant health-associated species in primary teeth also were significant in permanent teeth (data not shown). Species such as *Kingella oralis*, *Eubacterium saburreum* clone GT038, *Gemella morbillorum*, *S. cristatus*, and *Streptococcus intermedius* were found at a high level in healthy subjects and were at significantly reduced levels in the healthy enamel of diseased subjects in permanent teeth (Fig. 3). Other health-associated species in permanent teeth, such as *Streptococcus* sp. clone CH016, *Streptococcus* sp. group H6, *Leptotrichia* sp. clone DT031, *Eubacterium* sp. clone EI074, *Campylobacter showae*, *Fusobacterium nucleatum* subsp. *polymorphum*, and *Capnocytophaga sputigena*, were detected at reduced levels in plaque from white-spot or dentin lesions of diseased subjects compared to the levels detected in healthy subjects (Fig. 3). Previous studies support the presence of these species in the healthy oral cavity, which is different from those of oral disease (1, 10). Among the health-associated species, we also noted that *F. nucleatum* subsp. *polymorphum*, *E. saburreum* clone GT038, *Eubacterium* sp. clone EI074, *Eubacterium* sp. clone DO016, *Bacteroides* sp. clone BU063, *Leptotrichia* sp. clone DE084, *Leptotrichia* sp. clone DT031, *G. morbillorum*, *Streptococcus* spp., and *Kingella* spp.

were more pronounced in the secondary dentition than in the primary dentition (Fig. 2 and 3 and data not shown). Furthermore, we observed an overall greater diversity associated with caries in permanent teeth based on bacteria present at significant levels.

There were several disease-associated species with distinct bacterial profiles at each stage of caries progression (Fig. 4 and 5). In white-spot and dentin lesions, species like *S. parasanguinis* and *S. salivarius* were observed at high levels in both dentitions. In addition, *Corynebacterium* sp. clone AK153 and *A. gerencseriae* were detected at high levels in primary dentition, as were *Leptotrichia* sp. clone GT018 (data not shown), *Campylobacter gracilis*, and *Selenomonas* sp. clone EY04 in permanent dentition. The microflora of deep-dentin lesions was dominated by *S. mutans*, *Lactobacillus* spp., *Propionibacterium* sp. strain FMA5, and *Atopobium* genomospecies C1 (permanent) and *Bifidobacterium* spp. (primary) (Fig. 4 and 5). *S. mutans* seems to have a more dominating role in dentin and deep-dentin caries of primary teeth than in those of secondary teeth, in which additional species like *Propionibacterium* sp. strain FMA5 were commonly detected at higher levels (Fig. 4 and 5). All of these species are acid producers and may be involved in the etiology of caries. As illustrated in Fig. 4 and 5, these species were found at the highest levels only at the late stages of disease. Notably, 10% of the subjects with dental caries did not have detectable or low levels of *S. mutans*. Other studies also report about 10 to 15% of caries-active subjects that do not have detectable levels of *S. mutans*, and therefore the presence of *S. mutans* does not necessarily indicate caries ac-
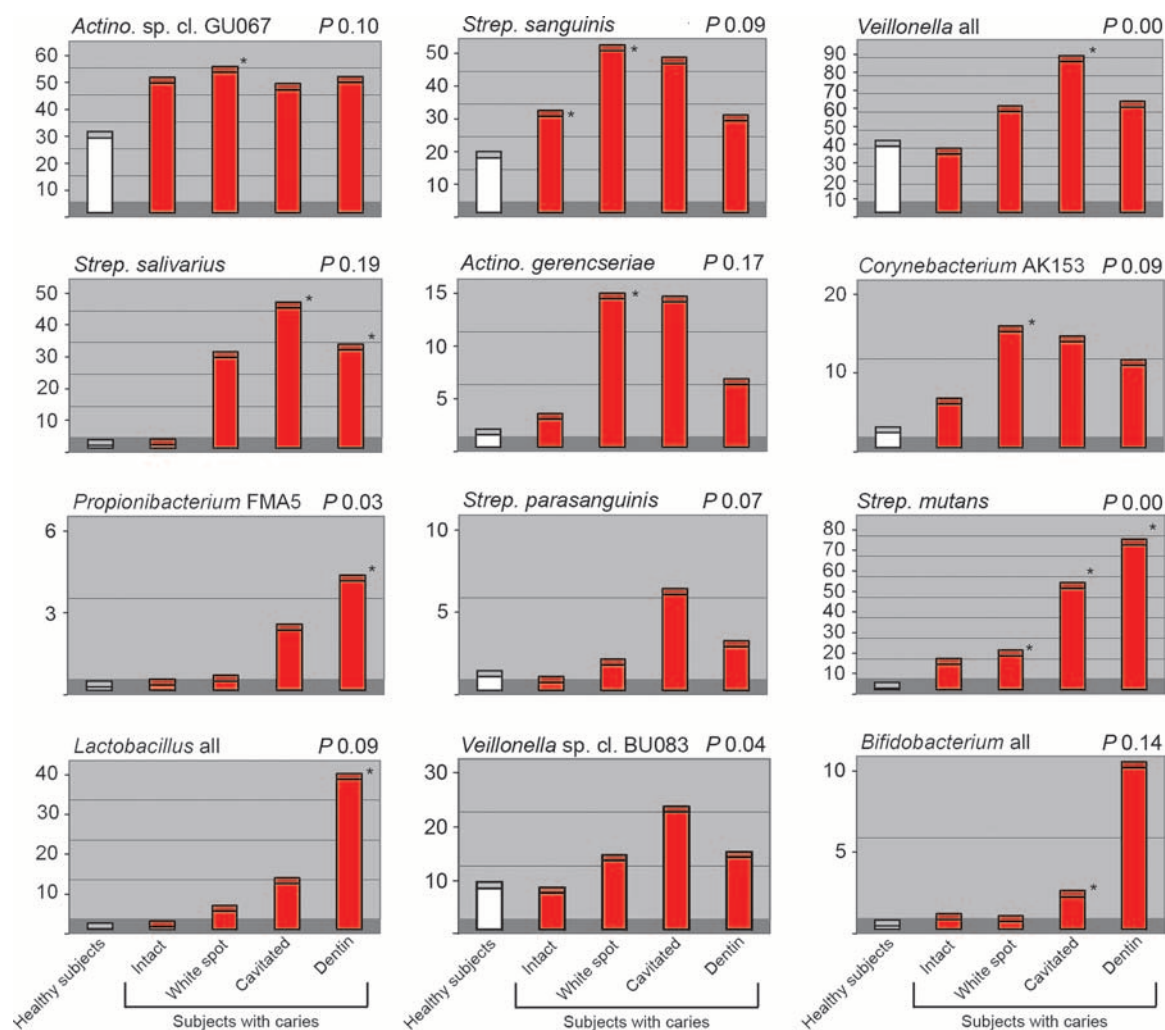
FIG. 4. Mean levels of caries-associated species or species groups in primary teeth determined by a reverse-capture checkerboard assay for 15 subjects with caries (red bars) and 14 healthy controls (white bars). *, $P \leq 0.05$ for pairwise comparisons of the healthy group to other groups by PROC MIXED. P indicates the overall P value comparing all groups and sites by PROC MIXED. *Strep.*, *Streptococcus*; *Actino*, *Actinomyces*; cl., clone.

tivity (4). These data clearly demonstrated that the bacterial etiology of caries involves other potential acid-producing species, some of which have not yet been cultivated. Further longitudinal studies with animal models are necessary to assess other cariogenic species in addition to *S. mutans* (13, 14). As in our study, additional studies based on molecular techniques performed by Munson et al. (29), Chhour et al. (8), and Corby et al. (10) found *S. mutans* and *Lactobacillus* spp. to be dominant in advanced caries. The results from the previous study on primary teeth (3) were confirmed, and additional species were detected according to the expanded panel of species. Our results also confirmed those of previous studies (3, 5, 8, 10, 15, 17, 25, 27, 29), indicating that a diversity of bacterial species, primarily gram-positive species, is involved in caries.

Veillonella spp., identified by a probe specific for all *Veillonella* spp., dominated the bacterial population in all stages, from intact enamel to deep-dentin cavities, in both the primary and secondary dentitions (Fig. 4 and 5). Other members of the *Acidaminococcaceae* family, such as *Selenomonas* spp., also

were well represented in healthy and diseased sites, as seen from both clonal and checkerboard analyses. These findings are consistent with the findings of Munson et al. (29) and Chhour et al. (8), who detected *Veillonella*, albeit at lower levels. These organisms use lactate as a metabolic carbon source (12) but have a limited ability to adhere to host tissue. *Veillonella* spp., however, participate in coaggregation with many oral bacteria, e.g., *Streptococcus* spp., *Eubacterium saburreum*, and *Actinomyces viscosus* (12). A number of observations have associated *Veillonella* spp. with caries. Bradshaw and Marsh (6) observed that *Veillonella dispar* was the most prevalent organism found after all glucose-pulsing regimens used, especially at low pH, and Noorda et al. (31) showed in mixed bacterial plaques with *S. mutans* and *Veillonella alcalescens* that the production of acid was higher than that in plaque containing only one of these species. The present study confirmed the coactive relationship between *Veillonella* spp. and acid-producing bacteria (28). *Veillonella* spp. also may be important for acid-producing bacteria in caries through nitrate reduction
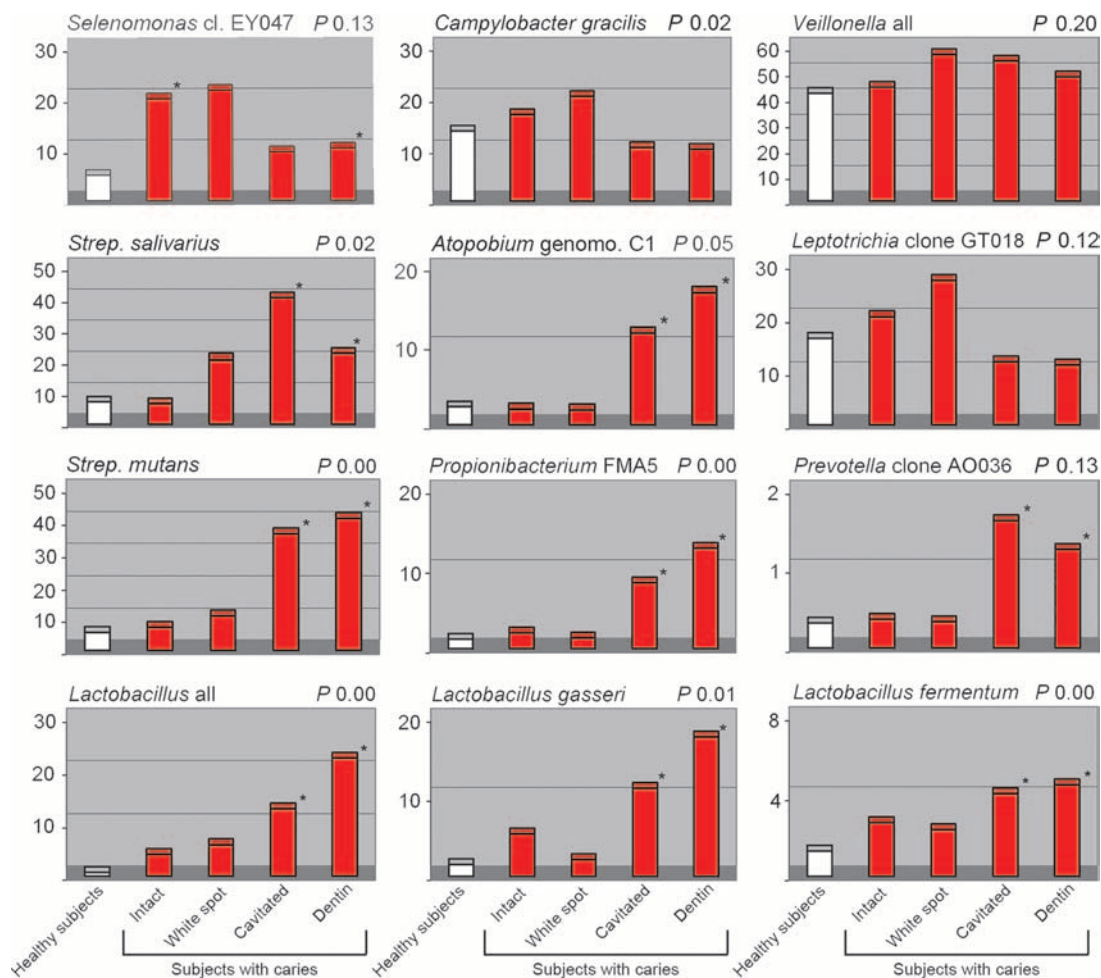
FIG. 5. Mean levels of caries-associated species or species groups in permanent teeth determined by a reverse-capture checkerboard assay for 36 subjects with caries (red bars) and 25 healthy controls (white bars). ∗, $P \leq 0.05$ for pairwise comparisons of the healthy group to other groups by PROC MIXED. P indicates the overall P value comparing all groups and sites by PROC MIXED. *Strep.*, *Streptococcus*; genomo., genomospecies.

(11). Silva Mendez et al. (36) demonstrated that acidity levels below pH 7 and low concentrations of nitrite (0.2 mM) caused the complete killing of *S. mutans*, with similar effects on the other organisms tested.

Actinomyces spp. play a major role in coaggregation interactions (9). Burt et al. (7) reported that *Actinomyces*-like organisms usually predominate in noncariogenic plaques with high levels of *S. mutans* streptococci and lactobacilli. *Actinomyces* spp. are heterofermenters but tend to become homolactic producers under anaerobic conditions (16), thus contributing to enamel demineralization. Strong coaggregation between *Actinomyces odontolyticus* and *Actinomyces israelii* with strains of either *Veillonella parvula* or *Prevotella prevotii* has been reported (35). Studies on coaggregation interactions between *Streptococcus* spp. and *Actinomyces* spp. have revealed the complementary adhesion-receptor mechanisms among the latter organisms (9). A large proportion of positively coaggregating pairs between either *Prevotella intermedia* ND8-9A or *Campylobacter gracilis* ND9-8A and strains of *Streptococcus*, *Gemella*, *Peptostreptococcus*, *Lactobacillus*, and *Actinomyces* indicate that the gram-negative obligate anaerobic rods play an

important role in the interactions leading to root caries (35). Nadkarni et al. (30) and Chhour et al. (8) found that novel and uncultured *Prevotella* and *Prevotella*-like bacteria dominate the diverse polymicrobial community in some cases of caries, suggesting an active role of *Prevotella* in caries progression. Furthermore, coaggregation between *Fusobacterium nucleatum* NT6-6A and six other bacterial species, i.e., *Streptococcus bovis* II/2 ND2-2, *Streptococcus constellatus* ND10-13A, *Streptococcus sanguinis* II ND7-3, *Lactobacillus acidophilus* ND7-2A, *C. sputigena* ND2-12A, and *P. intermedia* ND8-9A, shows that these bacteria have the ability to coaggregate with a large number of oral bacteria and possibly act as key organisms in dental plaque formation during the later stages of plaque maturation and modulation of the climax community (20, 21). With further evidence that a large number of bacterial species are involved in the progression of dental caries, the interactions within different bacterial communities representing different stages of the disease would be of considerable interest.

In light of recent efforts to develop vaccines specific for *S. mutans*, the study of the potential etiologic role of all species associated with caries and caries progression is essential. The

TABLE 3. Predominant species in bacterial profiles related to caries progression in primary and permanent dentitions[a]

Species detected in primary dentition					Species detected in permanent dentition				
Control	Intact enamel	White-spot lesion	Dentin lesion	Deep-dentin lesion	Control	Intact enamel	White-spot lesion	Dentin lesion	Deep-dentin lesion
Leptotrichia all[b]	*Actinomyces* sp. cl. GU067	*Veillonella* all	*Veillonella* all*	*Streptococcus mutans**	*Actinomyces* sp. cl. GU067	*Actinomyces* sp. cl. GU067	*Veillonella* all	*Veillonella* all	*Veillonella* all
Veillonella all	*Veillonella* all	*Actinomyces* sp. cl. GU067*	*Streptococcus mutans**	*Veillonella* all	*Fusobacterium nucleatum* subsp. *polymorphum*	*Veillonella* all	*Actinomyces* sp. cl. GU067	*Actinomyces* sp. cl. GU067*	*Actinomyces* sp. cl. GU067
Streptococcus anginosus	*Leptotrichia* all	*Streptococcus sanguinis**	*Actinomyces* sp. cl. GU067	*Actinomyces* sp. cl. GU067	*Veillonella* all	*Streptococcus sanguinis*	*Streptococcus sanguinis*	*Streptococcus salivarius**	*Streptococcus mutans**
Actinomyces sp. cl. GU067	*Streptococcus sanguinis**	*Leptotrichia* all	*Streptococcus sanguinis*	*Lactobacillus* all*	*Streptococcus sanguinis*	*Fusobacterium nucleatum* subsp. *polymorphum*	*Leptotrichia* sp. cl. GT018	*Streptococcus mutans**	*Streptococcus salivarius**
Capnocytophaga granulosa	*Streptococcus mitis**	*Streptococcus salivarius*	*Streptococcus salivarius**	*Streptococcus salivarius*	*Eubacterium* sp. cl. EI074	*Eubacterium* sp. cl. EI074	*Fusobacterium nucleatum* subsp. *polymorphum**	*Streptococcus sanguinis*	*Lactobacillus* all*
Fusobacterium all	*Capnocytophaga sputigena*	*Capnocytophaga sputigena*	*Veillonella* sp. cl. BU083	*Streptococcus sanguinis*	*Leptotrichia* sp. cl. DT031	*Leptotrichia* sp. cl. DT031	*Eubacterium* sp. cl. EI074	*Streptococcus mitis*	*Streptococcus sanguinis*
Streptococcus sanguinis	*Capnocytophaga granulosa*	*Streptococcus mitis*	*Streptococcus mitis*	*Streptococcus mitis*	*Fusobacterium animalis*	*Streptococcus mitis*	*Fusobacterium animalis*	*Fusobacterium animalis*	*Streptococcus mitis*
Campylobacter gracilis	*Streptococcus cristatus*	*Corynebacterium* sp. cl. AK153	*Selenomonas* sp. cl. EY047	*Veillonella* sp. cl. BU083	*Leptotrichia* all	*Leptotrichia* sp. cl. GT018	*Veillonella* sp. cl. BU083	*Veillonella* sp. cl. BU083	*Streptococcus anginosus*
Streptococcus cristatus	*Actinomyces* sp. strain B19SC	*Actinomyces gerencseriae**	*Leptotrichia* all*	*Actinomyces naeslundii* strain B19SC	*Actinomyces naeslundii* serotype II	*Selenomonas* sp. cl. EY047*	*Selenomonas* sp. cl. EY047	*Lactobacillus* all*	*Lactobacillus gasseri**
Capnocytophaga sputigena	*Streptococcus* sp. cl. CH016		*Actinomyces gerencseriae*	*Selenomonas* sp. cl. EY047	*Capnocytophaga sputigena*	*Capnocytophaga granulosa*	*Streptococcus mitis*	*Atopobium* genomospecies C1*	*Atopobium* genomospecies C1*

[a] %The results are based on the signal level in a reverse-capture checkerboard hybridization assay. *, $P \leq 0.05$ for pairwise comparisons of the healthy group to other groups by PROC MIXED tests. cl, clone.
[b] all, all species of the genus.

significance of our work is that the identification of additional caries pathogens provides alternative targets for biological interventions, and the identification of beneficial health-associated species could provide the basis for therapeutic interventions to establish caries-resistant microbial communities.

Although we have shown that specific bacterial species are associated with health, caries initiation, and caries production, there is subject-to-subject variation in the bacterial composition. Munson et al. (29) demonstrated that many species of *Lactobacillus* were found overall in carious lesions, but only one or two *Lactobacillus* spp. were detected in each lesion in a subject. We and others have shown that 10 to 20% of subjects with severe caries may not have detectable levels of *S. mutans* but rather exhibit other acid-producing species. Furthermore, in some carious lesions *S. mutans* may be a minor bacterial component of the dental plaque. These results support the ecological plaque hypothesis (26), which suggests that caries is a result of a shift in the balance of the resident microflora driven by changes in local environmental conditions, e.g., acid production by any number of species that cause tooth decay.

In summary, we have shown that half of the bacteria associated with dental caries have not yet been cultivated. Species in addition to *S. mutans*, e.g., species of *Veillonella*, *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, low-pH non-*S. mutans* streptococci, *Actinomyces*, and *Atopobium*, also may play an important role in caries production. *Actinomyces* spp. and non-*S. mutans* streptococci may be involved in the initiation of the disease. Several specific species are associated with health, while others are associated with caries. Bacterial profiles change with the progression of disease and differ from the primary to the secondary dentition. The present findings support the ecological plaque hypothesis in caries disease, in that changes in ecologic factors require different bacterial qualities and stimulate alterations in the bacterial composition. Further studies of the potential etiologic roles of these diverse bacterial communities, including additional and novel species, are recommended. Identified cultivable and not-yet-cultivable organisms might provide additional targets for caries intervention.

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